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Diabetes Obes Metab. 2018 September ; 20(Suppl 2): 28–50. doi:10.1111/dom.13378.**Biosynthesis, structure, and folding of the insulin precursor protein****Ming Liu, MD, PhD^{1,2}, Michael A. Weiss, MD, PhD^{3,4}, Anoop Arunagiri, PhD², Jing Yong, PhD⁵, Nischay Rege⁴, Jinhong Sun, MD^{1,2}, Leena Haataja, PhD², Randal J. Kaufman, PhD⁵, and Peter Arvan, MD PhD²**¹Department of Endocrinology and Metabolism, Tianjin Medical University General Hospital, Tianjin, China 300052²Division of Metabolism, Endocrinology & Diabetes, University of Michigan Medical School, Ann Arbor 48105 MI USA³Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis 46202 IN USA⁴Department of Biochemistry, Case-Western Reserve University, Cleveland 44016 OH USA⁵Degenerative Diseases Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92307 USA**Abstract**

Insulin synthesis in pancreatic beta cells is initiated as preproinsulin. Prevailing glucose concentrations, which oscillate pre- and post-prandially, exert major dynamic variation in preproinsulin biosynthesis. Accompanying upregulated translation of the insulin precursor include elements of the endoplasmic reticulum (ER) translocation apparatus linked to successful orientation of the signal peptide, translocation, and signal peptide cleavage of preproinsulin — all of which are necessary to initiate the pathway of proper proinsulin folding. Evolutionary pressures on the primary structure of proinsulin itself have preserved the efficiency of folding (“foldability”), and remarkably, these evolutionary pressures are distinct from those protecting the ultimate biological activity of insulin. Proinsulin foldability is manifest in the ER, in which the local environment is designed to assist in the overall load of proinsulin folding and to favor its disulfide bond formation (while limiting misfolding), all of which is closely tuned to ER stress response pathways that have complex (beneficial, as well as potentially damaging) effects on pancreatic beta cells. Proinsulin misfolding may occur as a consequence of exuberant proinsulin

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Conflict of interest

None declared.

biosynthetic load in the ER, proinsulin coding sequence mutations, or genetic predispositions that lead to an altered ER folding environment. Proinsulin misfolding is a phenotype that is very much linked to deficient insulin production and diabetes, as is seen in a variety of contexts: rodent models bearing proinsulin-misfolding mutants, human patients with Mutant *INS*-gene induced Diabetes of Youth, animal models and human patients bearing mutations in critical ER resident proteins, and, quite possibly, in more common variety type 2 diabetes.

Keywords

secretory protein biosynthetic pathway; polypeptide chain initiation; Sec61 translocon; disulfide-linked protein complexes; unfolded protein response

1 | INTRODUCTION

The dynamics of insulin biosynthesis and secretion has been investigated in recent studies of normal human volunteers who received isotopic amino acid bolus labeling and 45 minutes later underwent a standard 75-g oral glucose tolerance test [1]. These studies indicate that ~80% of the C-peptide released from beta cells over the ensuing 3.5 h is derived from ‘old’ (i.e., previously-made) hormone pools whereas only ~20% is newly-synthesized. As the storage pool of older insulin and C-peptide is vast, the small fractional contribution of new hormone nevertheless implies a ‘relative preference’ for the release of new insulin [2], particularly during the extended ‘second-phase’ of insulin secretion [1]. Significantly, these findings underscore the importance of insulin secretory pathway dynamics [3], as beta cell homeostasis requires the maintenance and repletion of the insulin granule storage pool. Such homeostasis is critically dependent upon the biosynthetic pathway [4–6] as well as granule turnover [7].

A pancreatic beta cell can readily synthesize 6000 preproinsulin molecules per second [8]. The process of folding new proinsulin includes injection of the nascent polypeptide into the lumen of the endoplasmic reticulum (ER) followed by signal peptide cleavage and formation of three proinsulin disulfide bonds that are evolutionarily conserved throughout the vertebrate insulin/IGF superfamily [9, 10]. Pancreatic beta cells folding this amount of proinsulin have significant ongoing ER stress response (e.g., spliced XBP1 protein) even in the absence of stimulation or addition of ER stressors [11]. After feeding, yet more proinsulin synthesis is required — and still more in insulin-resistant states such as pregnancy or obesity [12, 13].

The pathway of proinsulin folding has been analyzed in great detail by us and others, with a particular emphasis on how alterations in proinsulin disulfide bonding patterns in both rodent and human beta cells impact upon insulin production and secretion. Studies of *Akita* and *Munich* mouse models have shown that cysteine mutations in proinsulin interfere with proper disulfide bond formation. Such interference both aggravates ER stress and accounts for the onset of insulin deficient diabetes, culminating ultimately in beta cell loss [14, 15]. It is possible that even in the absence of such mutations, intracellular oxidative or reductive stress [16–18] could impair native disulfide pairing in proinsulin and in turn trigger the onset or progression of type 2 diabetes.

In this article, we bring together and unify four perspectives that span the features of preproinsulin biosynthesis including the earliest steps that deliver the protein to and through the ER membrane; structural features preserved in the proinsulin molecule to facilitate its folding to the native state in order to achieve ultimate biological activity; pathways of regulation of the ER environment that are designed to sense proinsulin folding load and to optimize successful proinsulin biosynthesis; and factors that drive proinsulin misfolding in the ER in spite of the beta cell's best efforts to limit this phenotype. These considerations lead us to the consequences of defects in any and all of the steps outlined herein: insulin production deficiency, beta cell ER stress, beta cell death, and diabetes.

2 | PREPROINSULIN BIOSYNTHESIS AND TRANSLOCATION ACROSS THE ER MEMBRANE

2.1 | ROUTES OF PREPROINSULIN ENTRY INTO THE ER

Newly synthesized preproinsulin is composed of the signal peptide followed by the entirety of proinsulin, including segments corresponding to the insulin B-chain, C-peptide, and insulin A-chain (Figure 1).

Preproinsulin biosynthesis is initiated in the cytosol. As a first step in insulin biosynthesis, newly synthesized preproinsulin must be guided by its signal peptide to the ER for translocation [19, 20]. In general, secretory preproteins can follow either co-translational and/or post-translational translocation routes into the ER. For preproinsulin, it had long been thought that only the signal recognition particle (SRP)-dependent co-translational mechanism was employed [21] in which translation is coupled to translocation into the ER [22–24]. Although this notion is compelling, emerging genetic and cell biological evidence indicates that preproinsulin translocation across the ER membrane is less efficient than previously thought [25–27]. Specifically, recent studies have shown that in isolated human islets and beta cell lines, during a 10-minute metabolic labeling period, about 5–15% of fully synthesized preproinsulin molecules are not translocated across the ER membrane. What's more, this measurement underestimates the problem. Although preproinsulin translation itself is expected to require < 20 seconds [28], when the pulse-labeling period was shortened to 300 seconds (i.e., ~15-fold longer than the translational requirement), up to 40% of completed preproinsulin nascent chains had not yet been translocated into the ER, demonstrating the marked and unexpected inefficiency of its co-translational translocation [26, 27, 29]. That fully synthesized but untranslocated preproinsulin molecules are unstable in the cytosol highlights the importance of their rescue via the post-translational translocation route.

In 2012 it was reported that whereas inhibiting expression of a subunit of SRP or the SRP receptor could impair SRP-dependent co-translational translocation, preproinsulin translocation could nevertheless continue, seemingly unaffected [30]. By contrast, knockdown of Sec62 (which has been shown to facilitate the post-translational mechanism) impaired preproinsulin translocation, suggesting that this route contributes significantly to the overall efficiency of preproinsulin translocation (Figure 2).

Indeed, an important fraction of translocation-dependent preproinsulin signal peptide cleavage was observed in beta cells to occur during the 10 minutes following completion of nascent chain biosynthesis [26], strongly supporting the notion that translocation of newly synthesized preproinsulin is co-translationally inefficient and is completed via a post-translational mechanism.

The pathophysiological significance of defects in preproinsulin translocation and signal peptide cleavage is highlighted by recent discovery of six diabetogenic preproinsulin signal peptide mutations (Figure 1) [25, 27, 29]. As for most other secretory preproteins, the preproinsulin signal peptide is comprised of three regions, a positively charged “n-region”, a hydrophobic “h-region”, and a more polar “c-region”; illustrative studies of the consequences of an n-region mutation are shown in Figure 3.

Diabetogenic preproinsulin mutations have been found in all three regions of the signal peptide, but interestingly, mutants in these three regions are associated with distinct diabetes phenotypes, suggesting that the mutations may utilize distinct mechanisms and perturb different underlying steps in preproinsulin biosynthesis — each nevertheless leading to beta cell failure and diabetes [29, 31, 32].

2.2 | INTRACELLULAR MACHINERY INVOLVED IN POST-TRANSLATIONAL TRANSLOCATION OF PREPROINSULIN

Whereas the intracellular components involved in co-translational translocation of secretory preproteins are well-established, those involved in post-translational translocation are less well studied. The Sec61 translocon is a common route for both co- and post-translational translocation. However, unlike for co-translational translocation, preproteins undergoing post-translational translocation appear to have additional requirements in order to efficiently engage the Sec61 translocon. To date, analyses of secretory preproteins that undergo post-translational translocation suggest that several cytosolic molecular chaperones, including Hsp70, TRC40, calmodulin, and Sec62/63 contribute to this pathway.

Hsp70 facilitates post-translational translocation, presumably by preventing aggregation of untranslocated preproteins — thereby maintaining translocation-competence [33–35]. The action(s) of Hsp70 might also account for the energy requirement of translocation. Proximity of calmodulin and TRC40 to signal sequences was demonstrated in crosslinking studies. Such binding is thought to maintain translocation-competence of preproteins and facilitate ER targeting and delivery. Inhibiting or downregulating either cytosolic protein was found to greatly impair post-translational translocation of preproteins [36, 37]. Additionally, two ER membrane proteins Sec62/63, are thought to be necessary for post-translational engagement with the Sec61 translocon [38, 39]. In the specific case of preproinsulin, Sec62 is the only component of the translocation machinery that has been experimentally confirmed to play a role in promoting post-translational translocation [30].

Another component that might contribute to the ER translocation of preproinsulin is the Translocon-Associated Protein complex (TRAP), which is also known as the Signal Sequence Receptor (SSR). A molecular neighbor of Sec61 [40, 41], TRAP was reported to facilitate initiation of protein translocation in a substrate-specific manner, especially for

preproteins with weak Sec61 interactions [42]. As preproinsulin is predisposed to inefficient translocation, it is possible that TRAP might enhance its efficiency, especially given that in a pancreatic beta cell line, mRNA encoding TRAP components are upregulated along with insulin mRNA in response to glucose stimulation [43]. Recently, a genetic polymorphism of TRAP α has been reported to be associated with type 2 diabetes, and with gestational diabetes [44, 45]. Additional studies will be needed to determine if the TRAP complex plays a role in insulin biosynthesis.

2.3 | INFORMATION INTRINSIC TO THE SEQUENCE OF PREPROINSULIN DETERMINES ITS ROUTE AND EFFICIENCY OF ER TRANSLOCATION

In addition to intracellular machinery, the biological properties of secretory preproteins are important factors in determining the translocation route chosen for entry into the ER. These intrinsic properties/signals include the length of the preprotein, the strength of the signal sequence, as well as structural information encoded in the preprotein beyond the signal peptide cleavage site [26, 30, 46, 47]. Among these factors, the length of the entire preprotein is one of the most important factors. Because SRP binding does not bring about complete translational arrest, smaller secretory proteins may easily be completed before SRP recognition or docking at the ER membrane, which leads to less efficient co-translational translocation: post-translational translocation is then enlisted “as a backup” mechanism. Indeed, a recent study compared the efficiency of translocation of hybrid proteins led by the preproinsulin signal sequence and followed by 70 – 300 amino acids. Co-translational translocation became gradually less efficient for preproteins containing fewer than 110 residues (the length of authentic human preproinsulin). Indeed, for preproteins of ~70 residues (thought to represent a minimal requirement for SRP binding), up to 70% of the molecules are not translocated into the ER upon completion of polypeptide synthesis [26]. This inefficiency tends to shift small secretory preproteins to the post-translational translocation route [48]. Given that many important cytokines and hormones (including insulin) are derived from small polypeptide precursors, the pathophysiological role of post-translational translocation may be far more important than previously realized.

The strength of the signal sequence that is recognized and bound by SRP also contributes to the choice of translocation route. Among the three regions of the signal sequence, a strong and undisrupted hydrophobic h-region appears to be the most important element in determining the strength of SRP binding. A short h-region, or one disrupted by polar or charged residues, may thus be subject to inefficient SRP binding and ER translocation [47, 49, 50]. The h-region of the human preproinsulin signal sequence contains at least 10 hydrophobic residues that would in principle provide a relatively high score for SRP binding. Therefore, we attribute the low co-translational translocation efficiency of preproinsulin primarily to its short length (which allows for rapid completion of synthesis with escape from the co-translational process) rather than a structural inability to be recognized by SRP. Despite this predisposition to inefficient co-translational ER targeting, untranslocated preproinsulin molecules nonetheless find their way to the ER membrane [26, 29, 31], presumably via the assistance of some of the machinery described in the preceding section.

Upon delivery to the ER membrane, the preproinsulin signal peptide needs to be properly oriented within the Sec61 translocon, such that the n-terminus of the signal peptide faces the cytosolic side of the ER membrane. This orientation allows the cleavage site of the signal peptide to be exposed to signal peptidase on the luminal side of the ER membrane. Charged residues in the n-region, or the charge gradient flanking the h-region, helps to orient the signal peptide to align itself with the complementary charge gradient existing within the Sec61 channel [29, 51]. This model is consistent with the fact that about 70% of the eukaryotic secretory preproteins contain positively charged residues in their signal sequence n-regions [26, 52]. Preproinsulin in particular has a highly conserved positive charged residue at the 6th residue of its signal sequence. In the case of the preproinsulin-R6C mutant, elimination of the n-region positive charge results in inefficient preproinsulin translocation, predisposing to beta cell failure and diabetes in humans carrying the mutation [25, 29]. Remarkably, the same diabetogenic mutation does not cause a detectable translocation defect when the mutant signal peptide is part of larger secretory preproteins, indicating that the defect caused by loss of the n-region positive charge is manifest only in small secretory preproteins, i.e., those (like preproinsulin) that are more dependent upon post-translational translocation [26].

3 | PROINSULIN STRUCTURAL FEATURES THAT ARE A CONSEQUENCE OF EVOLUTIONARY PRESSURES TO PRESERVE FOLDABILITY

3.1 | CLINICAL MUTATIONS AS PROBES OF FOLDABILITY

Dominant mutations in the human insulin gene can lead to pancreatic beta cell dysfunction and diabetes due to toxic folding of a mutant proinsulin (the human MIDY syndrome; see section 5.1, below). Whereas the majority of mutations introduce or remove a cysteine as highlighted above (leading in either case to an unpaired thiol), non-cysteine-related mutations in principle identify key determinants of folding efficiency. Studies of such mutations suggest that the evolution of insulin has been constrained not only by the structure and function of the mature hormone, but also by the susceptibility of its single-chain precursor to impaired foldability. Broad conservation of these non-cysteine residues suggests that rules of proinsulin foldability are shared by a family of single-chain factors (such as mammalian insulin like growth factors; IGF-I and II) and in part within the superfamily of invertebrate insulin-like proteins [53–55].

3.2 | MECHANISM OF DISULFIDE PAIRING

Oxidative folding of globular proteins has often been probed by chemical trapping of disulfide intermediates [56]. Application of this approach to proinsulin and related polypeptides (such as foreshortened single-chain analogs [57] and IGFs [58]) demonstrated transient accumulation of one- and two-disulfide intermediates [59–61]. Successive steps yield partial folds via a sequence of free-energy landscapes (Figure 4A). The broad basin in such landscapes enables multiple trajectories to the native state (bottom of funnels) from an ensemble of unfolded and partially folded states. This perspective extends the classical disulfide-centered view of protein-folding intermediates [62] to encompass general biophysical principles of protein folding as an ensemble of atomic-level pathways [63–65].

The landscapes “mature” from shallow to steep, enabling nascent proinsulin to fold stepwise on successive disulfide pairing. Preferred disulfide intermediates and their time course of accumulation, as defined by chemical trapping, thus corresponds to multiple folding trajectories on a progression of landscapes. Each landscape regulates the dynamics of accessible polypeptide conformations in the presence of a specific subset of disulfide bridges. MIDY mutations may in principle skew the shape of a particular landscape and/or block the transition from one to the next.

Refolding studies of native proinsulin (distinct from analogous studies of model systems and homologs) have been limited by aggregation, which has restricted such studies to be conducted at pH conditions above 9; this restriction has, at least in part, confounded interpretation in relation to mechanisms of folding and misfolding pertinent to conditions in the ER [67]. The disulfide pathways of mini-proinsulin and IGF-I are by contrast well characterized near neutral pH as investigated by several laboratories [59–61, 68–71]. A consensus pathway is shown in Figure 4B [68, 72–80]. In this scheme a pivotal role is played by A20-B19, the favored initial cystine. In the three-dimensional structure of native insulin, this disulfide bridge packs within a cluster of conserved side chains in the hydrophobic core, connecting the C-terminal α -helix of the A chain to the central α -helix of the B chain [81–83]. Because cystine A20-B19 is the only one-disulfide species to accumulate in the refolding of model- or homologous polypeptides [60, 61, 71], its pairing is envisioned to stabilize a specific folding nucleus [73, 80, 84].

3.3 | STRUCTURAL MODELS OF FOLDING INTERMEDIATES

The above findings motivated construction of analogs (of insulin, mini-proinsulin, and IGF-I) containing pairwise substitution of the other cysteines by Ala or Ser [68, 70, 72, 74–80]. Such analogs exhibit attenuated α -helix content as probed by circular dichroism (CD). Nucleation of native-like structure was nonetheless observed by isotope-directed ^1H - ^{13}C NMR spectroscopy in a disulfide-linked two-chain fragment of IGF-I containing cystine A20-B19: at low temperature the disulfide bridge native tethers α -helices within a native-like cluster of nonpolar side chains [80]. Mutations within this A20-B19-associated subdomain impair disulfide pairing in insulin chain combination and the biosynthesis of single-chain precursors in *Saccharomyces cerevisiae* [75, 84–87].

Remarkably, ^1H -NMR spectra of the above equilibrium models were found to exhibit a progressive chemical-shift dispersion with successive disulfide pairing, reflecting stepwise stabilization of structure in accordance with the successive landscape paradigm [68, 73]. After A20-B19 pairing, folding can proceed through several alternative channels. Oxidative refolding of mini-proinsulin, for example, exhibits subsequent rapid formation of cystine A7-B7 (lower pathway in Figure 4B) or slow pairing of A6-A11 (upper pathway). Although it is not intuitive why pairing of cysteines distant in the sequence (A7 and B7) is favored relative to pairing of nearby cysteines (A6 and A11), spectroscopic studies revealed that pairwise serine substitution of cystine A7-B7 destabilizes the structure of insulin more markedly than analogous removal of A6-A11 [84]. These findings suggest that nascent structure in the one-disulfide [A20-B19] intermediate either more effectively aligns Cys^{A7} and Cys^{B7} or more significantly impairs pairing of Cys^{A6} and Cys^{A11}. Either on-pathway

two-disulfide intermediate may interconvert with non-native disulfide isomers as off-pathway kinetic traps (central panel of Figure 4B).

3.4 | NON-NATIVE DISULFIDE PAIRING

The hazard to pancreatic beta cells posed by competing traps in the folding of proinsulin in the ER is considered in subsequent sections. The susceptibility of nascent proinsulin to forming non-native states is suggested by the aberrant refolding of a homologous polypeptide (IGF-I), which *in vitro* forms native and non-native disulfide isomers as alternative ground states (native IGF-I and IGF-*swap*) [59, 60]. Containing respective pairings [A20-B19, A7-B7, A6-A11] (native) and [A20-B19, A6-B7, A7-A11] (*swap*), the isomers exhibit similar core structures near the shared A20-B19 disulfide bridge [60, 88]. In a “mini-IGF” model, the relative stabilities of these disulfide isomers is influenced by the N-terminal segment of the B domain [89, 90]. In human proinsulin an homologous non-native isomer and yet another (with pairings [A20-B19, A11-B7, A6-A7]) forms in the presence of a chemical denaturant [83]. Whereas the stability of IGF-*swap* is similar to that of native IGF-I, the accessible non-native isomers of insulin and proinsulin are molten globules of marginal stability [82].

Evidence for the pathological relevance of non-native disulfide isomers of proinsulin and related polypeptides has been obtained in eukaryotic cell culture [91–95]. Non-native isomers of human proinsulin are readily resolved in an SDS-polyacrylamide gel based system (described further in Section 5, below). Amino acid substitutions in human proinsulin can enhance the fraction of mispairing in the ER [92, 93]. Because propensity to misfold in this assay does not correlate with effects of the substitutions on thermodynamic stability *in vitro*, its mechanism is not well understood.

3.5 | DETERMINANTS OF NATIVE DISULFIDE PAIRING

Non-cysteine-related MIDY mutations are broadly distributed in the B chain of insulin. “Hot spots” occur in the N-terminal segment (residues B1-B8), central α -helix (B9-B19), and adjoining beta-turn (B20-B23) together with its aromatic anchor (Phe^{B24}). An overview of key positions in these elements is provided in Figure 5. Each mutation site provides insight into a structural determinant of native disulfide pairing.

We will discuss specific examples in the direction C \rightarrow N as the C-terminal element of the B chain undergoes a change in conformation to serve a dual role in self-assembly and receptor binding [96].

The C-terminal segment of insulin (residues B24-B30) has attracted intensive investigation as it plays a central role in both homodimerization and receptor binding. Structure-function relationships pertinent to dimerization have been extensively investigated since elucidation of the crystal structure of insulin by Hodgkin and colleagues in 1969 [97]. An overview of this process is shown in Figure 6A.

The recent co-crystal structure of insulin bound to a fragment of the insulin-receptor (IR) ectodomain has provided evidence for induced fit within the C-terminal segment B-chain [100]. As predicted based on studies of “anomalous” insulin analogs [101–103], detachment

of the C-terminal beta-strand (B24-B27) enables its docking in a groove between the N-terminal L1 beta-helical domain of the IR α -subunit and its dimer-related C-terminal element, an α -helix denoted α CT. Pre-detachment of the B-chain beta-strand by a chiral perturbation (e.g., substitution by Phe^{B24} by D-Ala) was observed to enhance receptor binding at the expense of increased susceptibility to fibrillation [103]. Induced fit of the B chain may thus represent an evolutionary response to the danger of proteotoxicity: self-assembly of the closed state protects the hormone from fibrillation.

Phe^{B24} (shown in red in Figure 6A) is invariant among vertebrate insulins and IGFs. Its aromatic ring anchors the native B-chain beta-strand against the α -helical core of the insulin monomer. Such anchoring stabilizes the hydrophobic core and contributes to dimerization (and thus zinc hexamer assembly) [99]. Repositioning of Phe^{B24} enables induced fit on receptor binding [103] and engages with the receptor [104, 105]. Recent advances in the crystallographic analysis of the IR ectodomain as an inverted-V dimer (Figure 6B) has led to the identification of the primary hormone-binding elements, L1 and α CT [106, 107]. Repositioning of α CT on the surface of L1 requires repositioning of Phe^{B24} and displacement of residues B24-B30 as highlighted by superposition of the classical T state into a model complex (Figure 6C).

Whereas substitution of Phe^{B24} by Cys leads to a prototypical MIDY phenotype (presumably due to an odd number of cysteines, as in the *Akita* allele) [108], substitution by Ser was identified more than 30 years ago (by a team of investigators at the University of Chicago including the late D.F. Steiner, memorialized at this symposium) as a classical insulinopathy [111]. Associated with a native-like structure in NMR studies [112], this mutation led onset of diabetes in the proband's third decade of life rather than in infancy. Penetrance was variable in the pedigree.

Unlike classical insulinopathies Phe^{B25}→Leu (insulin *Chicago*) and Val^{A3}→Leu (insulin *Wakayama*), which markedly impair receptor binding [113, 114], Ser^{B24}-insulin retains substantial (although reduced) biological activity [115]. Studies of the proband indicated that the mutant and wild-type proinsulins were each processed and secreted into the bloodstream [115]. Biochemical and cell biological studies have nonetheless uncovered defects in the variant precursor's efficiency of folding, leading to aberrant induction of ER stress. Significantly, the level of ER stress was observed to be less than that associated with neonatal-onset MIDY mutations [115–117].

We speculate that heterozygous inheritance of the Ser^{B24} mutation contributes to a lifelong burden of ER stress in pancreatic beta cells; whether this burden leads to adult-onset diabetes presumably depends on genetic background, *i.e.*, co-inheritance of other genetic variants that mitigate or accentuate ER stress in beta cells.

B20-B22 β -Turn.—The super-secondary structure of the insulin B chain requires a β -turn between its central α -helix (residues B9-B19) and C-terminal β -strand. This turn contains conserved glycines at B20 and B23, which each exhibit a positive ϕ dihedral angle (ordinarily forbidden to L-amino acids). Whereas Ala substitutions impair the expression of mini-proinsulin in *S. cerevisiae* and impede chain combination, efficient disulfide pairing

can be rescued by D-amino acid substitutions [87]. A MODY mutation has been found at B23 and is expected to perturb pairing of cystine A20-B19. The structural mechanism by which a mutation at B22 (Arg→Gln; see above) causes a milder phenotype (analogous to Maturity-Onset Diabetes of the Young; MODY) is not apparent [117, 118].

Central α -helix.—Nascent folding of the B-chain α -helix was found in equilibrium models of the key [A20-B19] folding intermediate [68, 80] and subsequent two-disulfide intermediates [75, 78, 79, 84]. In these models Leu^{B11} and Leu^{B15} each contributed to clustering of nonpolar residues. MIDY mutations at these sites presumably block initial disulfide pairing and, should native disulfide pairing be achieved, perturb native structure and assembly. Indeed, Ala substitutions at B11 and B15 (although helicogenic in model peptides) impair the expression and secretion of mini-proinsulin in *S. cerevisiae* [86]. Whereas MIDY mutation Leu^{B15}→Pro would be expected to destabilize the entire central α -helix, even subtle substitutions in this helix have been found to impair insulin chain combination. A prominent example is provided by simple interchange of Leu^{B11} and Val^{B12}, predicted to perturb tertiary structure without net change in intrinsic helical potential [119]. It is not clear why substitution of Tyr^{B16} by His, predicted to project at the surface of this α -helix [120], may impair folding.

N-terminal segment.—In both the solution structure of insulin [72, 102, 121] and its crystallographic T-state [99, 122], residues B1-B6 are extended (asterisk in Figure 5) whereas B7-B10 comprise a β -turn adjoining the central α -helix. N-terminal residues contribute to the specification of the A7-B7 disulfide bridge *in vitro* [89, 90] and to the overall efficiency of proinsulin folding in cell lines [98]. In the classical TR transition among zinc insulin hexamers [123], this segment reorganizes as an α -helix to extend the central α -helix of the B chain. Although pertinent to insulin pharmacology [124], the physiological significance of the R state (if any) is not well understood.

Sites of MIDY mutation (His^{B5} and Gly^{B8}; broadly conserved among vertebrate insulins) have been well characterized [94, 125–127]. (i) In the native state His^{B5} packs within an inter-chain crevice, making one or more hydrogen bonds to carbonyl oxygens in the A chain. Diverse substitutions impair chain combination [94]; Arg^{B5} (which is observed in some non-mammalian insulins) is by contrast well tolerated, presumably through formation of analogous inter-chain hydrogen bonds as visualized in its crystal structure [127]. In mammalian cell culture substitution of His^{B5} by Asp blocks the folding and secretion of human proinsulin [94]. (ii) In the T state Gly^{B8} exhibits a positive ϕ dihedral angle (like Gly^{B20} and Gly^{B23} above). Whereas only Arg^{B8} and Ser^{B8} have been found to date in patients, any L-amino-acid substitution impairs chain combination; yield is by contrast rescued by D-substitutions [125]. Such stereospecificity suggests that the sign of the B8 ϕ angle contributes to the alignment or misalignment of neighboring Cys^{B7} for disulfide pairing.

Ser^{B8}-insulin retains substantial biological activity [125]. The solution structure of Ser^{B8} insulin as an engineered monomer retains native-like features yet reduced thermodynamic stability [94, 126]. Whereas decreased native-state stability can in principle contribute to misfolding, it is likely that a chiral main-chain perturbation at B8 may also impose a kinetic

block to disulfide pairing, such as via decreased stabilities of populated folding intermediates. Evidence for such kinetic blocks has been provided by cellular studies in which this and related mutations lead to formation of non-native disulfide isomers [94, 126]. Alternatively, it is possible that these residues contribute to interactions of nascent proinsulin with ER chaperones and the oxidative machinery of disulfide pairing [128]. Interestingly, the substantial biological activities of Ser^{B8}-insulin and other “non-foldable” analogs [94, 98, 126] indicates that residues required for efficient folding may be dispensable for the biological activity of the native state, once reached [129, 130].

3.6 | THE INSULIN A CHAIN

The relative paucity of non-cysteine-related mutations in the A domain of proinsulin among MIDY patients investigated to date may seem surprising, yet this may merely reflect an incomplete sampling of patients. Certainly, substitutions at the conserved sites Leu^{A16} and Tyr^{A19} do impair the efficiency of chain combination [86, 131, 132]. It is also possible that conserved non-cysteine residues in the A domain (Ile^{A2}, Asn^{A18}, Tyr^{A19}, Asn^{A21}) play a less critical role in disulfide pairing than do analogous internal residues in the B domain. Both explanations may have partial validity. On the one hand, studies of the mechanism of chain combination have shown that the N-terminal A-chain α -helix (residues A1-A8) is not required for disulfide pairing [81]. Such dispensability is in accord with a putative structural pathway in which segmental folding of this α -helix is a late event (Figure 4B). On the other hand, the efficiency of chain combination is exquisitely sensitive to substitutions of core residue in the C-terminal A-chain α -helix (Leu^{A16} and Tyr^{A19}; purple spheres in Figure 5) [131, 133], and in fact mutation of Leu^{A16} has recently been recognized as a cause of human MIDY [134]. Thus we would expect that continued genetic screening of MIDY patients may well uncover additional non-cysteine-related A-chain mutations.

4 | DYNAMIC REGULATION OF THE ER ENVIRONMENT IN RESPONSE TO SUCCESSFUL AND UNSUCCESSFUL PROINSULIN BIOSYNTHESIS

4.1 | UNFOLDED PROTEIN RESPONSE

It is important to understand how an increase in proinsulin folding load can cause ER stress, and conversely how ER stress conditions affect proinsulin folding. The most ancient, conserved ER stress response (unfolded protein response, or UPR) is signaled through activation of the Inositol-Requiring Enzyme 1 (IRE1), a protein kinase and endoribonuclease (described below). Two more recent evolutionary developments in ER stress signaling include the Protein kinase-like Endoplasmic Reticulum Kinase (PERK) and the transcription factor ATF6 [135–137].

It is generally believed that both active and inactive subpopulations of the three ER stress sensors co-exist in normal cells, with the proportion of the active subfractions linked to cellular physiology and secretory pathway activity. The inactive subpopulations of ER stress sensor proteins are thought to be engaged in interactions with the ER protein chaperone BiP. An increased load of misfolded proteins in the ER effectively recruits BiP, thereby promoting a shift in BiP distribution away from PERK, IRE1 and ATF6 [138, 139], which is

strongly correlated with an increase in the size of the active subpopulations for these stress sensor proteins (Figure 7).

Individually, the PERK-eIF2 α phosphorylation pathway serves as a translational brake that can help to alleviate ER protein load upon ER stress. There are multiple lines of evidence implicating inadequacy of PERK signaling as a possible trigger for proinsulin misfolding. *Perk* gene deletion in the mouse recapitulates many of the defects of the human Wolcott-Rallison syndrome including diabetes associated with degeneration of β -cell mass after birth [140, 141]. In these studies, ER distention, a morphological indicator suggesting ER stress, was observed by electron microscopy in pancreatic beta cells from *Perk* null mice. Normoglycemia can still be maintained with a 95% deletion of PERK within the adult beta cell population in mice that have not been challenged with high fat diet or other causes of insulin resistance [141]. Nevertheless in the setting of insufficient PERK activity, the rate of glucose-stimulated proinsulin synthesis is enhanced (although such a conclusion has been recently challenged [142]), which is most consistent with the notion that loss of PERK kinase activity results in a loss of homeostatic maintenance of newly-synthesized proinsulin.

Activated PERK phosphorylates the α -subunit of the eukaryotic translation initiation factor 2 (eIF2 α) at Ser residue S51, leading to rapid, reversible inhibition of mRNA translational initiation [143]. The heterotrimeric GTPase eIF2 is required to bring initiator methionyl tRNA^{Met} to the 40S ribosome for AUG initiation codon recognition [144]. Phosphorylation of eIF2 α to generate eIF2 α -P inhibits the GDP/GTP exchange reaction of the eIF2B complex, thereby preventing eIF2 guanine nucleotide recycling which accompanies the initial step of protein synthesis [143, 145]. The S51A phosphorylation mutant in the *eIF2 α* locus was introduced into mice that, globally, cannot phosphorylate the mutant eIF2 α by any of the four known eIF2 α kinases [145]. Homozygous mutant mice die perinatally due to hypoglycemia and defective hepatic gluconeogenesis. However, heterozygous *eIF2 α -S51A* mice survive without a significant phenotype. Importantly, when fed a HFD that triggers insulin resistance, these mice develop beta cell failure and overt diabetes [146]. In addition, since the eIF2 α -S51A mutation in beta cells recapitulates all the effects of PERK deletion in mice and humans [147], the findings support the notion that PERK acts through eIF2 α -P, one important consequence of which is attenuation of proinsulin (and other protein) accumulation in the ER, thereby limiting further ER stress. In the setting of unusually increased levels of proinsulin misfolding, CHOP protein (controlled primarily via the PERK branch of the UPR pathway) can contribute to beta cell death associated with unsuppressed protein synthesis and increased generation of intracellular reactive oxygen species ROS [144, 148]. Importantly, deletion of the ER stress-induced proapoptotic gene *Chop* delayed onset of hyperglycemia in the *Akita* mouse [149] and suppressed β -cell failure in response to insulin resistance in diet-induced obese mice and *db/db* mice *db/db* mice [150]. To summarize, for the PERK branch of the UPR, although free fatty acids, cytokines and high glucose are proposed to cause β -cell failure, analysis of mice with *eIF2 α -S51A* mutation or *Perk* deletion [140, 141] supports the notion that dysregulated proinsulin handling in the ER is sufficient to initiate β -cell failure.

The two other branches of the ER stress response, namely, the IRE1 α and ATF6 α pathways, also impact proinsulin folding by controlling ER proteostasis [137, 151, 152]. Although

there are α - and β -alleles of both IRE1 and ATF6 in the mammalian genome, IRE1 α is ubiquitously expressed (whereas IRE1 β is more narrowly expressed, particularly in intestinal epithelial cells) and ATF6 α contributes more broadly to UPR signaling (whereas ATF6 β has a far more limited activity in beta cells [153]).

IRE1 α auto-phosphorylation activates a cytosolically-oriented domain of the protein encoding an endoribonuclease that initiates non-conventional splicing of *Xbp1* mRNA [151], removing a 26-base intron that leads to a shift in the translational open reading frame to produce a highly active bZip transcription factor, XBP1s. XBP1s activates genes encoding ER protein translocation, ER chaperones, lipid biosynthetic enzymes and ERAD functions [154, 155], all of which are thought to be needed for glucose-stimulated insulin secretion and cell adaptation and survival in response to physiological demand [156].

Activation of the ATF6 α pathways is highly analogous to the SREBP activation pathway in that upon release from BiP, unsequestered ATF6 traffics to the Golgi complex where it gets cleaved by S1P and S2P proteases to produce a 50 kDa cytosolic fragment that translocates to the nucleus to serve as a potent transcriptional activator on genes that have complementary or overlapping functions to those activated by XBP1s [152]. Studies implicate an important role of ATF6 in β -cell function and survival [157, 158]. Collectively, the XBP1s and ATF6 pathways serve a central function to maintain a productive ER protein folding environment, to increase ER-associated degradation (ERAD) to degrade misfolded proinsulin [151, 152], and to help maintain the islet population of mature differentiated beta cells [159, 160]. Indeed, cells deficient for *Ire1a*, *Xbp1* or *Atf6a* are defective in ERAD [161–163]. The importance of ERAD in normal cellular physiology has recently been reviewed elsewhere [164].

4.2 | THE PROTEIN DISULFIDE ISOMERASE (PDI) FAMILY OF ER OXIDOREDUCTASES, AND ERO1 AS AN UPSTREAM ER OXIDASE

The ER maintains a highly oxidizing intraluminal environment [17], which is considered crucial for proper proinsulin folding [165]. The protein disulfide isomerase (PDI) family of ER-resident proteins are strongly implicated in the oxidative folding of secretory proteins including proinsulin [166, 167]. Pancreatic islets are enriched in mRNA for PDIA1 [168], along with other abundant PDI-like oxidoreductase family members, such as ERp72 (PDIA4), P5 (PDIA6), ERp57 (PDIA3), PDIp (PDIA2), and ERp44, and may be further enriched in islets experiencing ER stress [169–172]. Although PDIA1 is important for correct proinsulin oxidative folding *in vitro* [173, 174], it is currently unknown if PDI plays any essential role for proinsulin oxidation *in vivo* [175], although it might play additional roles in the fate of proinsulin in pancreatic beta cells [176].

To the extent that PDI proteins oxidize proinsulin or other substrates, their own catalytic cysteine thiols need to become re-oxidized in order to promote further cycles of substrate oxidation. In pancreatic beta cells, there is abundant expression of ERO1 α , which is ubiquitous to all cells, and there is additional selective high expression of ERO1 β [177], which can also serve as a major oxidase for beta cell ER oxidoreductases [178, 179]. ERO1 β regulates susceptibility to ER stress and can be induced by proinsulin flux [180]. *In vivo*, *Ero1 β* deficiency produces a phenotype of increased proinsulin entrapment in the ER, which

is associated with lower beta cell insulin content and reduced insulin secretion [178]. Nevertheless, not all PDI family members require ERO1 to catalyze their re-oxidation (although such re-oxidation is thought necessary to allow PDI-mediated substrate oxidation to continue), suggesting the possibility of alternative oxidative recycling pathways for PDI family members [181, 182].

4.3 | ER REDOX BALANCE AND THE POTENTIAL THERAPEUTIC UTILITY OF ANTIOXIDANTS

A substantial quantity of hydrogen peroxide (H_2O_2) is formed as a by-product of the ERO1-catalyzed disulfide-formation pathway [183], although it is not clear that these particular ROS forms meaningfully escape the ER to attack other cellular compartments [184], and to the extent that they do, this might actually be beneficial for insulin gene expression [185]. Chemically, H_2O_2 in the ER forms at least in part via the action of ERO1 proteins, which utilize thiol-mediated electron transfer from members of the PDI family of oxidoreductases to molecular oxygen (Figure 8, and see below).

Alternatively, ROS may also be generated as a consequence of a futile cycle of oxidizing and reducing glutathione (GS-SG and GSH, respectively [181]). As a consequence, pancreatic beta cells, bearing the large load of proinsulin that must assemble three disulfide bonds per molecule, may be more susceptible to ER protein misfolding in response to overactivity of various oxidative enzymes [186]. In theory, pancreatic beta cells could utilize the activity of peroxiredoxin-4 (PRDX4) to consume H_2O_2 in the ER in order to make additional proinsulin disulfide bonds and resist ER stress [187]; however, evidence to date has suggested a limited expression of PRDX4 in rodent islet beta cells [188], although it becomes induced in islets of rodents upon high fat diet [189].

Interestingly, under certain circumstances, antioxidant treatment can effectively reduce ER stress and subsequent cell death [190]. This effect may be partially attributed to improved ER protein folding efficiency in cells with reduced oxidative stress. Feeding with a diet supplemented with the chemical antioxidant butylated hydroxyanisole (BHA), or deletion of the ER stress-induced proapoptotic gene *Chop*, significantly improved glucose homeostasis and restored beta cell function in mice bearing beta cell deletion of *p58^{ipk}* (discussed below), the ER co-chaperone for BiP which is linked to proinsulin misfolding [148]. Intriguingly, antioxidants (i.e. BHA) can improve proinsulin folding and reduce apoptosis under conditions of ER stress [148]. These data provide support that in the face of ER stress, restoration of cellular redox balance with appropriate antioxidants can improve beta cell viability and function, and that a critical aspect of the improved function is an improvement in proinsulin folding in the ER lumen [148].

Collectively, these data underscore that appropriate levels of ROS generation and scavenging are necessary for proper proinsulin folding, while excessive protein synthesis and unfolded ER protein accumulation can cause an imbalance of ROS and uncontrolled oxidative stress, predisposing to beta cell death [148, 150, 191].

4.4 | ER QUALITY CONTROL OF PROINSULIN ANTEROGRADE EXPORT

BiP is the most abundant ER protein chaperone and it functions by binding to hydrophobic patches [192, 193] in unfolded/misfolded proteins [194, 195]. Therefore, to a first approximation, the extent of proinsulin misfolding can be monitored by the extent of its interaction with BiP [146]. Importantly, such association with BiP [196, 197] (as well as other ER-resident peptide-binding proteins such as GRP94 [198]) participates critically in the mechanism of retention of unfolded proteins within the ER lumen (ER quality control).

Importantly, in high fat diet (HFD) fed mice that do not bear any *INS* gene mutation, heterozygosity for eIF2 α -S51A, which results in exuberant proinsulin biosynthesis, results in a dramatic increase of proinsulin interaction with BiP [146], underscoring a critical interaction between gene (heterozygous eIF2 α -S51A) and environment (HFD) in predisposition to beta cell failure. On the other hand, p58^{IPK} is a molecular co-chaperone that is proposed to stimulate the ATP hydrolysis activity of BiP, which is necessary for the cyclical binding/release of BiP to its client proteins [199]. Indeed, *p58^{IPK}/DNAJc3* deleted mice develop oxidative stress in beta cells, with loss of beta cell mass, leading to diabetes at about 6 weeks of age [200, 201]. Although there is still debate on the ER localization of P58^{IPK} [201, 202], biochemical study supports its direct involvement in assisting *Akita* mutant proinsulin folding, together with PDIA6 [167]. Interestingly, *p58^{IPK}* mutation in humans is also associated with diabetic complications and multi-systemic neurodegeneration [203].

4.5 | ER CALCIUM BALANCE

Last but not least, proinsulin folding in the ER is affected by ER calcium homeostasis. The ER is a dominant storage organelle for calcium in metazoans [204]. The most abundant ER resident proteins, including GRP94, BiP/GRP78, PDI, calreticulin, and others, are influenced by calcium binding, and these chaperones may in turn affect proinsulin folding. In addition, pancreatic islets exhibit glucose-dependent cytosolic calcium oscillations in electrical activity, which are coupled with ER calcium changes [205, 206].

Calcium accumulation in the ER lumen is maintained by the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) family of calcium pumps. The SERCAs are encoded by three genes (*Serca1*, *Serca2*, and *Serca3*), and their variety and activity are further diversified by the existence of splice variants and responses to ER stress [207]. SERCA2b and SERCA3 are the most abundant isoforms expressed in pancreatic beta cells [208, 209] and are largely responsible for maintaining the ER free calcium concentration in the physiological range of up to ~500 μ M [210]. SERCA pumps and store-operated calcium channels restore ER luminal calcium upon the rapid release of ER calcium that occurs when beta cells are acutely stimulated. Although impaired SERCA function has been implicated in T2D models [211], mice with *Serca3* deletion have normal basal glucose levels and glucose-stimulated insulin secretion. Indeed studies of *Serca3* deleted murine islets model [208, 209] have led to several insightful conclusions: 1) Basal cytosolic calcium is mainly set by mechanisms other than SERCA pumps; 2) The initial phase of increased cytosolic calcium can be taken up by the ER in a SERCA-dependent manner; 3) During the later phase of cytosolic calcium oscillations, the ER pool modulates the frequency and amplitude of these

oscillations. Interestingly, altered cytosolic calcium oscillation from *Serca3* deleted islets is associated with increased insulin secretion in response to glucose stimulation [208, 209]. Thus the ER calcium pool may influence insulin secretory release in response to nutrients. Finally, although theoretically possible [206], it is unknown if ER Ca^{2+} concentrations may fluctuate under physiological and pathophysiological conditions to a level that could affect proinsulin folding under the influence of calcium-sensitive chaperones and oxidoreductases [204, 212].

Inositol 1,4,5-tri-Phosphate Receptors (IP_3Rs) are a main source of acute calcium release from the ER. IP_3Rs are encoded by three genes (*Itpr1*, *Itpr2* and *Itpr3*), with splicing variants that display a range of IP_3 binding affinities, with activation that affects cytosolic calcium concentration [213]. The IP_3Rs form tetrameric channels that are distributed throughout the ER in clusters, and these clusters are considered calcium release “hot-spots” [204, 214]. Another class of calcium release channels is composed of the ryanodine receptors (RyR), which are encoded by three genes (*Ryr1*, *Ryr2*, and *Ryr3*). RyRs are not highly expressed in pancreatic islets, and may contribute little to the ER Ca release in beta cells.

Although calcium homeostasis in the ER of pancreatic beta cells is far more complex topic than what is described above, a basic understanding is that: 1) Free calcium concentration in the ER lumen regulates ER chaperones and oxidoreductases to facilitate proinsulin folding, and 2) The free calcium in the ER lumen is also expected to have an effect on cytosolic calcium bursts that fine-tune insulin granule exocytosis in response to nutrients such as glucose and amino acids.

5 | PROINSULIN IN DIABETOGENIC CONDITIONS: MISFOLDED STATES IN THE ER

In spite of the fact that proinsulin biosynthesis is tightly coordinated with metabolic demand via physiological ER stress response (especially the activities of IRE1 α /XBP1 to regulate expression of much of the ER machinery necessary for successful proinsulin and insulin biosynthesis [11, 156, 215]) — it is increasingly recognized that proinsulin is highly susceptible to misfolding [9, 216]. When added to this underlying predisposition, coding sequence mutations such as occur in *Akita* proinsulin trigger a cascade of misfolding, blockade of ER export, intracellular accumulation of the mutant proinsulin, and a defect in the production and secretion of co-expressed wild-type insulin [217, 218]. Although it was initially thought that misfolded proinsulin (as occurs in *Akita* mice) cannot promote formation of aberrant intermolecular disulfide bonds [219], this conclusion was inconsistent with the demonstration of an increase in disulfide-bonded proinsulin aggregates in *Akita* islets [14]. It is now thought that aberrant intermolecular disulfide-bonded proinsulin is not only a notable feature in *Akita* mouse islets, but also occurs in WT mouse islets [219], underscoring that WT proinsulin already has a propensity to misfold within the ER of pancreatic beta cells [93, 165, 220].

5.1 | HETEROZYGOUS PROINSULIN MUTATIONS ARE A (RARE BUT SEVERE) CAUSE OF PROINSULIN MISFOLDING

Proinsulin misfolding underlies the syndrome known as Mutant *INS* gene-induced Diabetes of Youth (MIDY) [32, 221], a human version of the disease seen in *Akita* mice. Clinically, the majority of patients with this syndrome develop life-long insulin-deficient diabetes mellitus that typically begins in the neonatal period (although – depending upon the mutation – may occasionally be initiated later in life [25]). As expected in neonatal diabetes, it is not uncommon to observe very rapid clinical development of pancreatic beta cell failure [222]. Although patients with MIDY do not have insulin autoantibodies [223, 224], the disease has often been labeled clinically as type 1 diabetes [225]. Notably, all MIDY patients are heterozygotes, indicating the presence of co-expressed WT proinsulin. Recently, the first large animal (porcine) model of the proinsulin-C(A7)Y mutation has been developed [226, 227] which recapitulates all of the features of MIDY as elucidated both in *Akita* mice and humans [25, 216]. It is known that the detrimental effects of MIDY mutant proinsulin expression is dose-dependent and related to the mutant : WT ratio [218, 226, 228–230]. In *Akita* mice, the mutant proinsulin protein represents 36% of total newly-synthesized proinsulin translation product [231]; in transgenic MIDY pigs, total *INS* mRNA is ~44% mutant and ~56% WT; and in humans with MIDY the mutant : WT ratio is believed to be 50% : 50%. The transgenic MIDY pigs first develop fasting hyperglycemia in the diabetic range between 2–3 months of life, and at 4.5 months of life when fasting glucose is > 300 mg/dL, fasting circulating insulin and C-peptide levels are low, corresponding with decreased pancreatic insulin, decreased beta cell mass, and a markedly expanded and fragmented beta cell ER [226, 227]. From there, the animals progress to develop ketosis as detected by a further decrease of insulin immunostaining and a drastic loss of conventional islet structures (see Figure 9), similar to what is reported in *Akita* mice [232].

In addition to aberrant intramolecular proinsulin folding, the mutant proinsulin-C(A7)Y forms aberrant disulfide-linked protein complexes [231] that include (and co-precipitate) WT bystander proinsulin [230] in the ER [233], thereby blocking WT proinsulin intracellular transport through the secretory pathway and causing beta cell ER stress [231] while also impairing downstream insulin production and insulin secretion [231], leading to diabetes [232]. Other MIDY mutants generate much the same pathological phenotype [32, 234] although the intricacies of intramolecular disulfide mispairing differ for individual MIDY mutants [165].

5.2 | LIPOTOXICITY AND PROINSULIN MISFOLDING

Free fatty acids have pleiotropic effects on pancreatic beta cells. In the near term, fatty acids such as palmitate can augment insulin synthesis and secretion [235]. However, chronic fatty acid exposure significantly damages beta cells, and together, high glucose and fatty acid exposure can synergize (negatively) to trigger beta cell ER stress [236]. Clinically, increased levels of saturated free fatty acids, especially palmitate, tend to predict an increased risk of type 2 diabetes [237].

Palmitate feeding to beta cells has been found to acutely increase cytosolic calcium and decrease ER calcium, leading to rapid phosphorylation of PERK, as well as other indicators

of ER stress [238]. In a pancreatic beta cell line fed palmitate for 6 h, proinsulin (fluorescently-tagged) was found to shift its intracellular distribution to become accumulated within the ER, with evidence of expansion of the ER compartment [239]. Although it remains to be examined directly, these findings are highly suggestive of the possibility of fatty acid (palmitate) exposure leading to proinsulin misfolding.

One recent study has suggested that the beta cell ER stress generated by fatty acids might also be mediated through ER calcium depletion via calcium leak (to the cytosol) through the ER translocon [240]. Interestingly, the protein synthesis elongation inhibitor anisomycin has been found to be beta cell-protective and prevents loss of ER calcium upon palmitate treatment, presumably by keeping the Sec61 translocation channel occupied with ribosomes. While anisomycin also enhances IRS-2 phosphorylation, making it is difficult to exclude other indirect effects, it is worth noting that using a protein synthesis inhibitor to simply decrease the rate of proinsulin synthesis should help to improve proinsulin folding efficiency. As a proof of concept, decreased insulin gene expression has similarly been shown to significantly alleviate ER stress and enhance Akt phosphorylation (and even promote beta cell proliferation) – and these effects that accompany decreased proinsulin synthesis can be observed independently from the use of any general protein synthesis inhibitor or other drugs [241].

Recent studies in *db/db* mice have suggested that suppression of Elovl6 (a microsomal enzyme that converts saturated and mono-unsaturated C-16 fatty acids to C-18 species) can enhance islet insulin content leading to improved insulin secretion and glycemic control [242]. This is clearly an interesting new system in which to explore potential lipotoxic effects on proinsulin biosynthesis and folding. Further, several compounds have been identified that not only enhance survival of primary human beta cells (as well as rodent beta cell lines) treated with tunicamycin (that inhibits N-linked protein glycosylation in the ER with consequent ER stress) and palmitate, but also enhance glucose-stimulated insulin secretion in these stressed cells [243]. These and other candidate compounds need further examination for their potential ability to decrease proinsulin misfolding, in addition to their salutary effects on ER stress and ER stress-mediated cell death.

5.3 | CYTOKINES AND PROINSULIN MISFOLDING

Free fatty acids may also promote inflammation in islets, potentially via TLR4 activation and macrophage stimulation resulting in the local release of inflammatory cytokines including interleukin-1 β (IL-1 β) [244], as well as by other potential mechanisms [245]. The signaling from IL-1 β , and interferon- γ (IFN- γ) and IFN- α , have been suggested to perturb the ER microenvironment [246], especially by lowering free calcium in the ER lumen [247] (perhaps by inhibitory effects on lumenally-directed SERCA-2 or other ER calcium pumps [248–250]). As described in preceding sections, the proinsulin folding process in the ER depends significantly on the ER microenvironment to facilitate both entry of newly-synthesized (pre)proinsulin into the ER as well as the activity of folding chaperones and oxidoreductases that operate within a regulated redox range and a controlled level of ER luminal calcium [156]. Protection from cytokine-mediated toxicity has been reported in beta

cells bearing ectopically-expressed mitochondrial catalase to selectively limit mitochondrial ROS production [251].

Nitric oxide generation in or around islets has also been suggested to be linked to cytokine response with lowered beta cell ER luminal calcium, leading to beta cell apoptosis [252], but the significance of these observations — at least in human beta cells — is still not very clear [253]. Cytokine-mediated lowering of ER luminal calcium levels is indeed thought to be linked to ER stress, but while the underlying mechanism of calcium lowering and the applicability of the findings for human islets are ongoing areas investigation [249], a recent study suggests that inadequate upregulation of *Spry2* (which is downstream of the ER stress-mediated PERK pathway) could limit SERCA-2 activity and also lead to diminished *INS* mRNA expression [254].

Human beta cells cultured short-term with IL-1 β and IFN- γ have been found to exhibit unabated proinsulin synthesis (with perturbed proinsulin-to-insulin conversion leading to increased unprocessed proinsulin secretion [255]). Remarkably, however, after 48 h of inflammatory cytokine exposure, there is a dramatic loss of islet insulin content and diminished glucose-stimulated insulin secretion [253]. Proinsulin misfolding under these conditions is a topic that clearly needs further investigation, but others have already gone ahead to employ an IL-1 β receptor antagonist (as a potential therapy for type 2 diabetes) with a beneficially increased ratio of insulin : proinsulin in the circulation [256].

Interestingly, if cytokines do alter the ER microenvironment in a manner that triggers chronic, severe proinsulin misfolding leading to hyperactivation of the IRE1 α limb of the ER stress response, this apparently can trigger a signaling cascade that can lead ultimately to still more IL-1 β secretion from pancreatic beta cells [257]. If so, it is conceivable that inflammatory cytokine exposure could trigger a dangerous positive feedback loop in which perturbation of the beta cell ER could ultimately result in the progressive generation of more inflammatory cytokines, worsening beta cell function and survival. An additional consequence of inflammatory cytokine stimulation may include ER stress-mediated formation and release of proinsulin-containing exosomes that could stimulate antigen-presenting cells, leading to beta cell autoimmunity [258].

5.4 | AGING AND PROINSULIN MISFOLDING

Aging cells are thought to accumulate misfolded proteins through a functional decline in the machinery necessary for maintenance of protein homeostasis [259]. Glucose intolerance and Type 2 diabetes incidence also tend to increase with age and this can be attributed primarily to an age-related decline in insulin secretion [260–264]. The detrimental effects of aging do not appear as evident in mouse models [265]. Nevertheless, isolated islets from NOD mice (a model of autoimmunity) display age-dependent increases in parameters of ER stress and morphologic alterations in ER structure by electron microscopy [266] (as well as an increase in islet levels of cytosolic hsp90 [267]), which accompany a progressive defect in insulin secretion [268]. While it is unclear that such age-dependent predispositions also exist in wildtype mice, the immunohistochemical detection of insulin in the islets of wildtype Sprague-Dawley rats has been noted to decline significantly with age [269]. Direct

evaluation of proinsulin misfolding as a function of age remains a fertile area for further investigation.

5.5 | HYPERGLYCEMIA AND PROINSULIN MISFOLDING

From meal-to-fasting-to-meal, new insulin synthesis is dramatically increased or decreased by changes in prevailing blood glucose levels [270], primarily through up- and down-regulation of preproinsulin mRNA translation (along with that of proinsulin processing enzymes [271], although this coordinate expression may not persist in type 1 diabetic human pancreata that generate proinsulin but no detectable insulin or C-peptide [272].) Ordinarily, the translational control of proinsulin biosynthesis is designed to replenish the beta cell secretory granule pool size; however, there are many pathophysiological examples of a discord between a high magnitude of proinsulin biosynthesis that is not matched by a similar steady-state number of stored insulin secretory granules [273, 274]. Such may be the case in type 2 diabetes. Although it has been reported that only a few percent of pancreatic insulin may be released for each meal, granule release iterated over time may deplete the storage pool, as reported in leptin receptor-deficient *db/db* diabetic mice [274] in which hyperglycemia has been reported as the predominant factor underlying the defective beta cell phenotype [275]. In such mice, proinsulin biosynthesis is markedly increased, the proportion of intracellular proinsulin is also increased, and the fraction of beta cell area occupied by ER is doubled [274]. Under these conditions, the quantity of misfolded proinsulin is expected to be increased. Proinsulin synthesis in WT pancreatic islets increases shortly after exposure of islets to high glucose levels, and with that biosynthetic increase is observed an increase in nonnative intramolecular disulfide isomers (see Figure 10, left two lanes).

Additional stimulation of proinsulin synthesis is observed in islets in which PERK is absent or inactive — exposure of such islets to elevated ambient glucose levels increases proinsulin biosynthesis still further, making nonnative intramolecular disulfide isomers still more abundant (Figure 10, right two lanes; and similar observations of proinsulin misfolding have been made in islet beta cells bearing eIF2 α mutation that prevents its phosphorylation by PERK [146]). It is thus of particular interest that *db/db* diabetic mice also show little evidence of PERK being active even in the face of ongoing high level proinsulin synthesis [274]. And despite this active proinsulin biosynthesis, these are not conditions in which intracellular insulin levels are maintained — in fact there is dramatic beta cell insulin depletion, indicating significant functional defects along the pathway of proinsulin folding, trafficking, processing, and storage [276].

Indeed, in WT mice trying to dispose of high glucose (continuous intravenous infusion of 277 μ moles/h) over a 4-day period, blood glucose is observed to rise within the normal range by 5–10 mg/dL each day, while circulating insulin in the bloodstream begins to decline between days 2, 3, and 4 [277]. As extracellular glucose levels rise higher still, beta cells can continuously sustain a very high level of proinsulin biosynthesis [278]. Only at the highest extracellular glucose levels consistent with full-blown diabetes (e.g., >500 mg/dL) do beta cells (at least rodent beta cells) finally show a tendency to decrease insulin mRNA accompanied by diminished proinsulin translation [279] as well as the formation of ubiquitylated intracellular protein aggregates [280]. While it is uncertain what proteins may

accumulate in these aggregates, it is certain that newly-synthesized proinsulin itself, when translated under high glucose conditions, forms protein complexes [220]. Thus all evidence points to the notion that sustained hyperglycemia is a major pathological trigger for proinsulin misfolding.

6 | FUTURE PERSPECTIVES

Even as we uncover more details about the earliest events of preproinsulin biosynthesis and folding, many questions remain. Regarding our considerations of the post-translational translocation process, more information is needed about the cytosolic chaperones that prevent untranslocated preproinsulin aggregation in order to maintain its post-translational translocation-competence. We also hope to learn how cytosolic preproinsulin is guided to the ER membrane. Further, more information is needed to identify all the components at the ER membrane that first engage nascent cytosolic preproinsulin and presents the signal peptide n-region positive charge to the Sec61 translocon. Perhaps the most important work still needed on preproinsulin translocation is to uncover the pathophysiologic implications of the different translocation mechanisms. Specifically, it has been suggested that ER translocation efficiency may be regulated under physiological conditions, and dysregulated under pathological conditions. It is possible that under ER stress, translocation efficiency may decrease, potentially re-routing some secretory (and membrane) proteins for cytosolic degradation — a process termed “pre-emptive quality control” [281–283]. Although pre-emptive quality control might reduce protein load into the ER, it might also result in proteotoxicity if these proteins are not efficiently cleared from the cytosol [281].

The above considerations culminate in a key question: how long is an untranslocated preproinsulin molecule allowed to spend in the cytosol before it is deemed unable to complete translocation and is targeted for degradation, and what mechanism initiates the switch from translocation to degradation? Once this is known, it should be possible to test the question of whether ER stress causes accumulation of undegraded preproinsulin molecules and/or contributes to beta cell failure in type 1 or type 2 diabetes [160, 216, 266, 284, 285]?

Regarding our considerations of the proinsulin folding pathways, we are impressed by the remarkable experiments of nature, defined as MIDY mutations, to highlight structural determinants of foldability. Together, biochemical, biophysical and cell biological studies suggest that sequence variation in insulin is enjoined by the intersection of multiple inter-dependent constraints (Figure 11). Yet salient overall questions remain, particularly with respect to the mechanism of native disulfide pairing. The structural differences between the native fold and competing meta-folds containing non-native disulfide bridges can be subtle. For example, mini-proinsulin (which contains a peptide bond between residues B29 and A1) refolds *in vitro* more efficiently than proinsulin [286] and yet in *S. cerevisiae* forms predominantly a metastable disulfide isomer that passes ER quality control and is secreted. These data suggest that the ER folding machinery of a eukaryotic cell can selectively target folding into a non-ground-state conformation; *i.e.*, global thermodynamic stability is not the critical parameter, which can thus lead to states of “stealth” misfolding, including the secretion of a protein caught in a kinetic folding trap. One future perspective is the

development of refined assays to reveal such stealth proinsulin misfolding in pancreatic beta cells.

In considering the ER folding environment for proinsulin, there are many unanswered important interesting questions that require further study. In particular, why do generalized perturbations of ER proteostasis — in both human cases and murine genetic models [140, 141] — especially disrupt beta cell function and survival? One plausible explanation may be that as opposed to other cell types, glucose also stimulates proinsulin mRNA translation via activation of PP1 phosphatase to dephosphorylate eIF2 α [287]. As a result, increased accumulation of proinsulin in the ER may activate the UPR to a level that may promote beta cell dysfunction. Specifically, post-prandial glucose excursions promote metabolic activity in beta cells that may generate ROS that accompanies ER stress [288]. Beta cells are particularly sensitive to ROS as they express low levels of antioxidant enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase [289], which may explain why antioxidants can limit beta cell failure in several mouse models [147, 148, 156].

Another interesting unanswered feature of disorders of ER stress response is the apparent link between type 2 diabetes and neurodegeneration, such as is seen animals with loss of function mutations in the ER chaperone *DNAJc3/p58^{IPK}*. Intriguingly, many nucleotide polymorphisms in genes identified for neurodegenerative diseases, such as the involvement of PERK in progressive supranuclear palsy (PSP) [290] and in Alzheimer's disease, may also impact beta cell function. These observations suggest a common sensitivity to ER dysfunction in both neurons and beta cells. Alternatively, could insulin signaling contribute to neurodegeneration? In the coming years, tissue-specific and temporally-controlled genetic models should help to enlighten this field.

Regarding our consideration of proinsulin misfolding under diabetogenic conditions, there also remains much to learn. There is strong reason to believe that the time during which proinsulin is most susceptible to misfolding is immediately upon translocation, prior to the completion of chaperone- and oxidoreductase-assisted folding. During this period, wild-type proinsulin in healthy beta cells is known to form disulfide-linked proinsulin complexes [291]. Ultimately it will be important to develop assays that can establish whether such complexes that persist for more than a few minutes after synthesis are true folding intermediates from which native proinsulin is still recoverable — or do they represent folded states that are irretrievably “off pathway”?

Finally, additional critical studies are still needed to directly examine nonmutant proinsulin folding status in “garden variety” type 2 diabetes, both in rodent models and in humans. Based on the foregoing considerations in this review, we present an hypothesis in Figure 12. We suggest that misfolded proinsulin beyond a critical threshold level impairs the ER export of bystander proinsulin molecules, similar to what occurs in MIDY. A decrease of proinsulin ER export, or absolute proinsulin levels (through ERAD and autophagy) limits proinsulin available for insulin production. The ensuing insulin deficiency leads to hyperglycemia which itself — along with other potential factors, promote further proinsulin misfolding, resulting in an unfortunate positive feedback loop (Figure 12). We hypothesize that the

accumulation of misfolded proinsulin contributes to beta cell ER stress, and in conjunction with the glucolipotoxicity of diabetes, contributes to pancreatic beta cell death.

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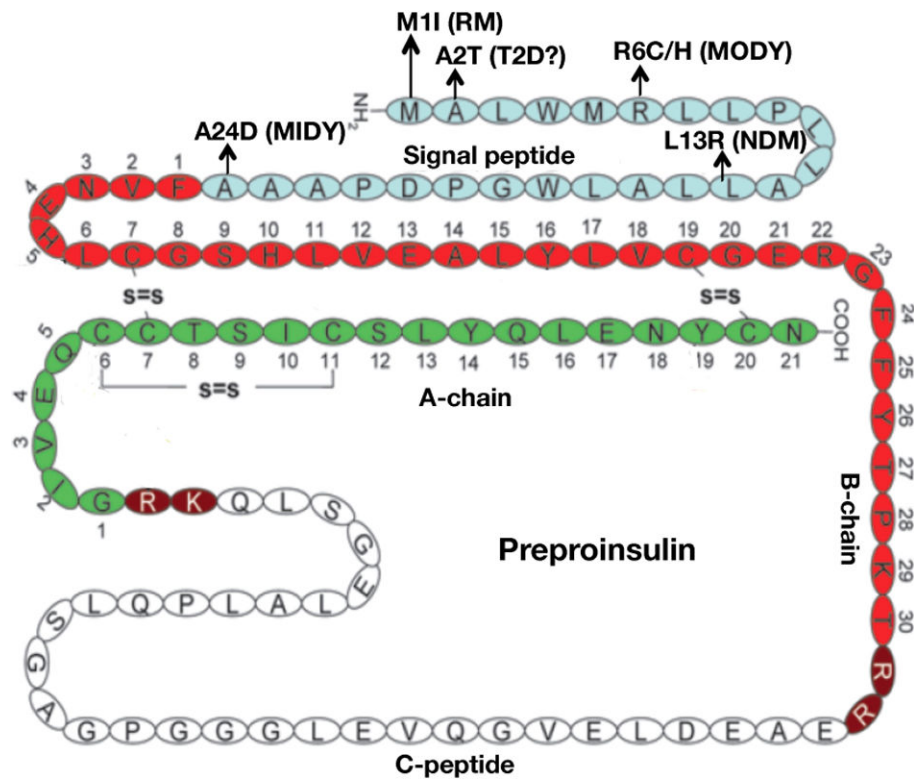


Figure 1. Human preproinsulin and its signal peptide mutations associated with diabetes in humans.

Preproinsulin is comprised of the signal peptide (light blue), insulin-B chain (red), C-peptide (white), and insulin-A chain (green). Signal peptide mutations reported to cause diabetes in humans are indicated. RM: recessive mutation; T2D: type 2 diabetes; MODY: maturity onset diabetes of the young; NDM: neonatal diabetes; MIDY: mutant *INS*-gene induced diabetes of youth.

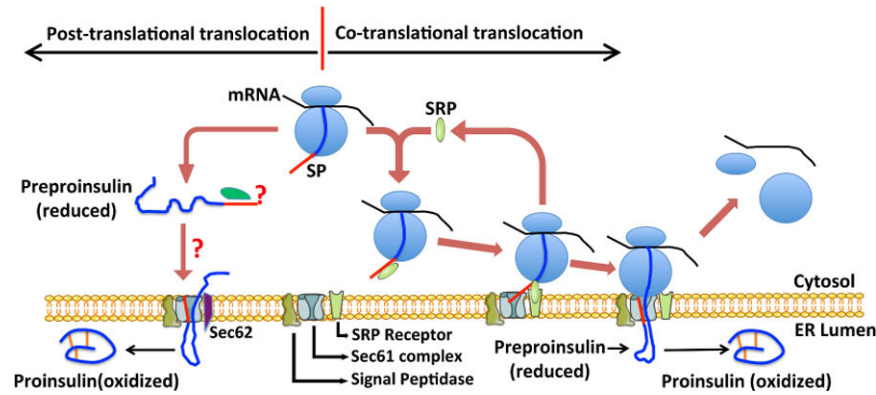


Figure 2. Preproinsulin translocation across the ER membrane.

Newly synthesized preproinsulin molecules can be translocated into the ER through both SRP-dependent co-translational translocation and SRP independent post-translational translocation. In co-translational translocation, SRP recognizes and binds the preproinsulin SP emerging from the ribosomes, forming SRP-ribosome-preproinsulin complexes that interact with SRP receptor on the ER membrane, thereby targeting the nascent preproinsulin to the Sec61 translocon. For post-translational translocation, although Sec62 is reportedly involved, the chaperones maintaining translocation competence of fully-synthesized preproinsulin in the cytosol, and its mechanism of delivery to the ER membrane, have not yet been identified. This figure is reprinted with modification, with permission from Vitamins and Hormones (reference 19).

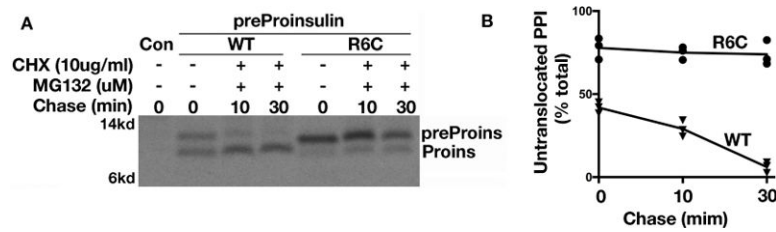


Figure 3. The n-region positive charge in preproinsulin signal peptide is critical for efficient post-translational translocated of preproinsulin.

A. 293T cells transfected with plasmids encoding preproinsulin wild-type (WT) or R6C mutant were pulse-labeled with ^{35}S -Met/Cys for 5 min followed by 0, 10, or 30 min of chase in the presence of 10 μM MG132 and 10 $\mu\text{g/ml}$ cycloheximide (CHX). Post-translational translocation of preproinsulin-WT and R6C were analyzed by immunoprecipitation, SDS-PAGE, and phosphorimaging. **B.** The signal-uncleaved and signal-cleaved forms of preproinsulin were quantified; the signal-uncleaved fraction is shown. Reprinted with permission from the J. Biol. Chem. (reference 26).

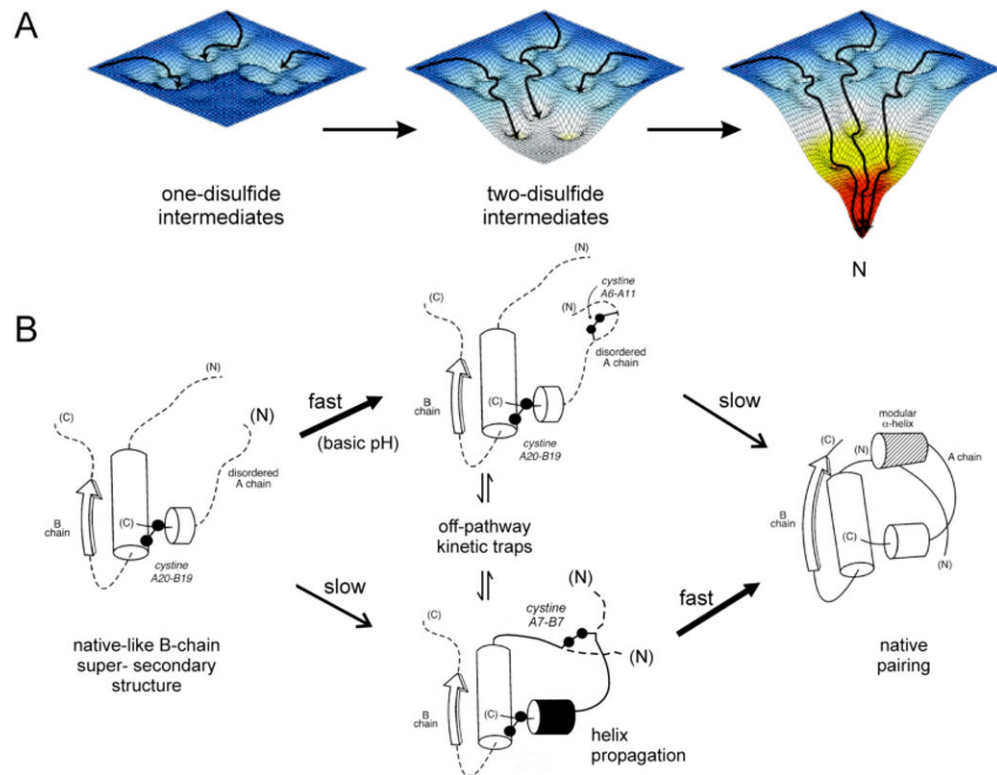


Figure 4. Energy landscape paradigm.

(A) Successive disulfide pairing enables a sequence of folding trajectories on ever-steepier funnel-shaped free-energy landscapes. (B) Preferred pathway of disulfide pairing. Initial formation of cystine A20-B19 (left) is directed by a nascent hydrophobic core comprising the central B-domain alpha-helix (residues B9-B19), part of the C-terminal B-chain beta-strand (B24-B26), and part of the C-terminal A-domain alpha-helix (A16-A20). Alternative pathways mediate successive disulfide pairing (middle panel) leading in turn to the native state (right). The mechanism of disulfide pairing is perturbed by clinical mutations associated with a monogenic syndrome of DM due to toxic misfolding of the variant proinsulin in the ER. Figure is adapted from Ref [66]; panel A is adapted from an image kindly provided by J. Williamson.

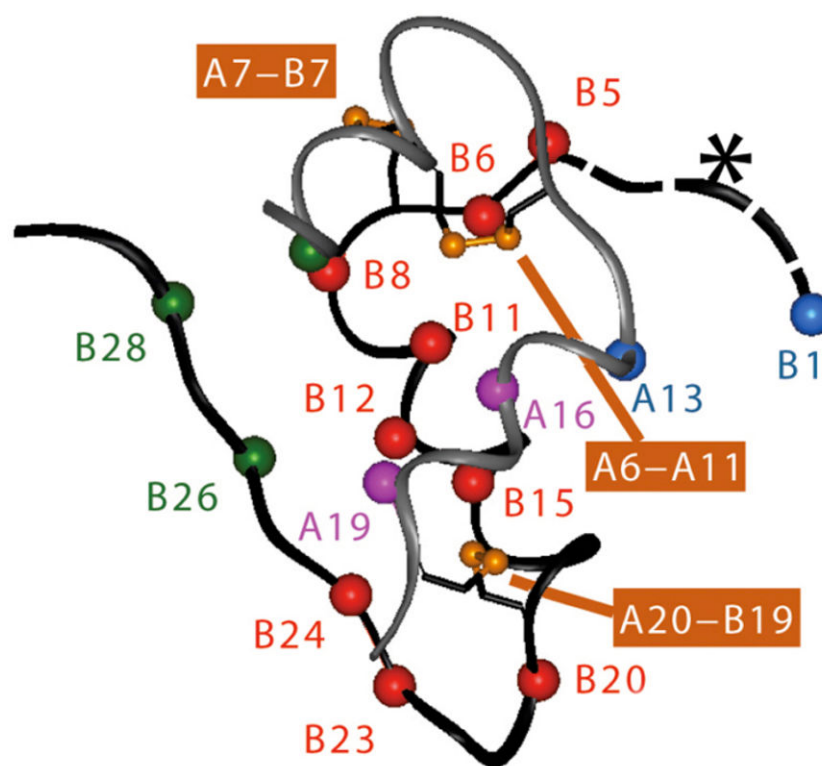


Figure 5. Critical sites governing the foldability of proinsulin are widely distributed in insulin. Asterisk indicates N-terminal segment of the B chain, which promotes foldability but is dispensable in the mature hormone [98]. Conserved side chains in or adjoining the C-terminal -helix of the A chain (Leu^{A16} and Tyr^{A19}; C_α purple spheres) and at multiple sites in the B chain (C_α red spheres) impair insulin chain combination in accord with studies of mutant proinsulins in mammalian cell lines and the distribution of clinical non-cysteine mutations in the insulin gene. Contacts between the side chains Phe^{B1} and Ile^{A13} (C_α blue spheres), although not well ordered in the native state, contribute to the cellular foldability of proinsulin. Residues Ile^{A2}, Tyr^{B26}, and Pro^{B28} (C_α green spheres) contribute to the structure and stability of the native state but are not required for efficient disulfide pairing in chain combination. Disulfide bridges are as indicated (orange). Coordinates were obtained from Protein Databank file 4INS and correspond to molecule 1 of the classical 2-Zn insulin hexamer [99].

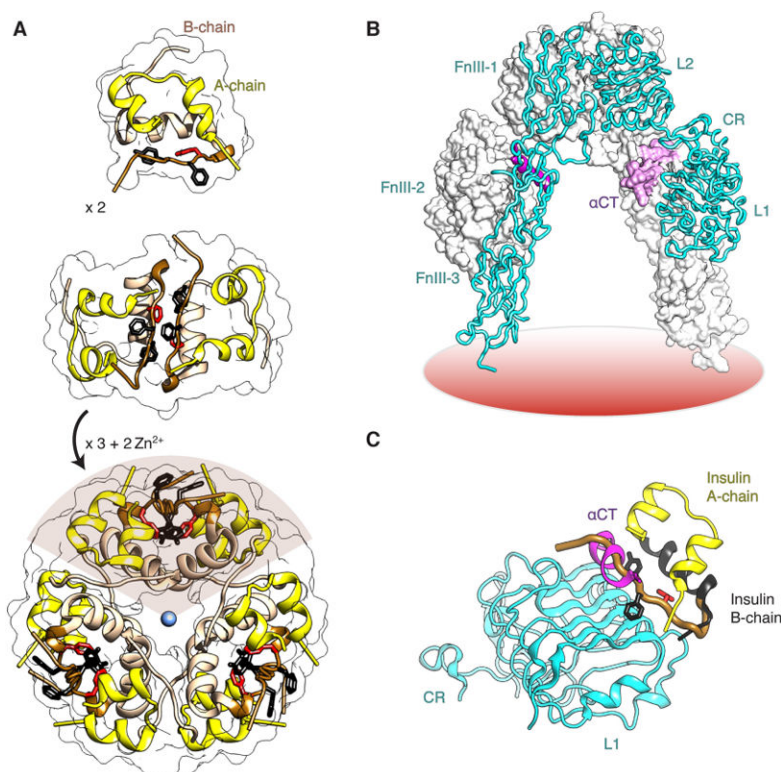


Figure 6A. Structure and assembly of insulin with key roles of Phe^{B24}.

Assembly of zinc insulin hexamer. The monomeric hormone (A- and B chains, *top* panel) forms zinc-free dimers via anti-parallel association of B-chain α -helices and C-terminal β -strands (brown, *middle* panel); two zinc ions then mediate assembly of three dimers to form classical hexamer (T₆, *bottom* panel). The A chain is shown in *yellow* (ribbon), and the B chain in *light brown* (B1–B19) or *brown* (B20–B30). Conserved aromatic residues Phe^{B25} and Tyr^{B26} are shown as *black sticks* whereas Phe^{B24} is *red*. The Zn²⁺ ion is depicted in *blue*. The figure was in part modified from reference [109] with permission of the authors. Coordinates were obtained from 4INS.

Figure 6B and 6C. Structure of IR receptor ectodomain. (B) Λ -shaped IR ectodomain homodimer. One protomer is shown as a ribbon (*labeled*), and the other as molecular surface. Domains are: L1, first leucine-rich repeat domain; CR, cysteine-rich domain; L2, second leucine-rich repeat domain; FnIII-1–3, respective first, second and third fibronectin Type III domains; and α CT, α -chain C-terminal segment. (C) Model illustrating insulin in its classical free conformation bound to Site 1 of the microreceptor (L1–CR + α CT 704–719; designated IR) [104, 105]. L1 and part of CR are shown in *cyan*, and α CT in *magenta*. Phe^{B24}, Phe^{B25} and Tyr^{B26} are as in **Figure 6A**. The B chain is shown in *dark gray* (B6–B19); the position of the *brown* tube (residues B20–B30) would lead to steric clash between B26–B30 and α CT. The figure was in part modified from references [109] and [110] with permission of the authors. Coordinates were obtained from PDB entries 4OGA, 2DTG, and 3W11.

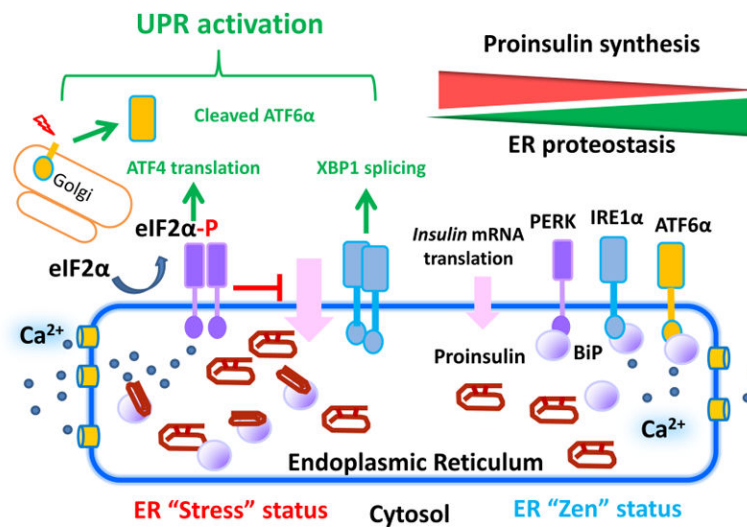


Figure 7. Illustration of ER stress response pathways and their activation by excessive proinsulin synthesis.

Increased synthesis and abundance of newly-made proinsulin correlates with increased BiP binding to proinsulin, which is associated with dimerization and activation of PERK and IRE1 α proteins, and translocation of full-length ATF6 α to the Golgi complex for proteolytic processing. Subsequent translation of ATF4 and spliced XBP1, together with the liberated cytosolic domain of ATF6 α , activate major transcriptional limbs of the stress response designed to restore proteostasis in the ER.

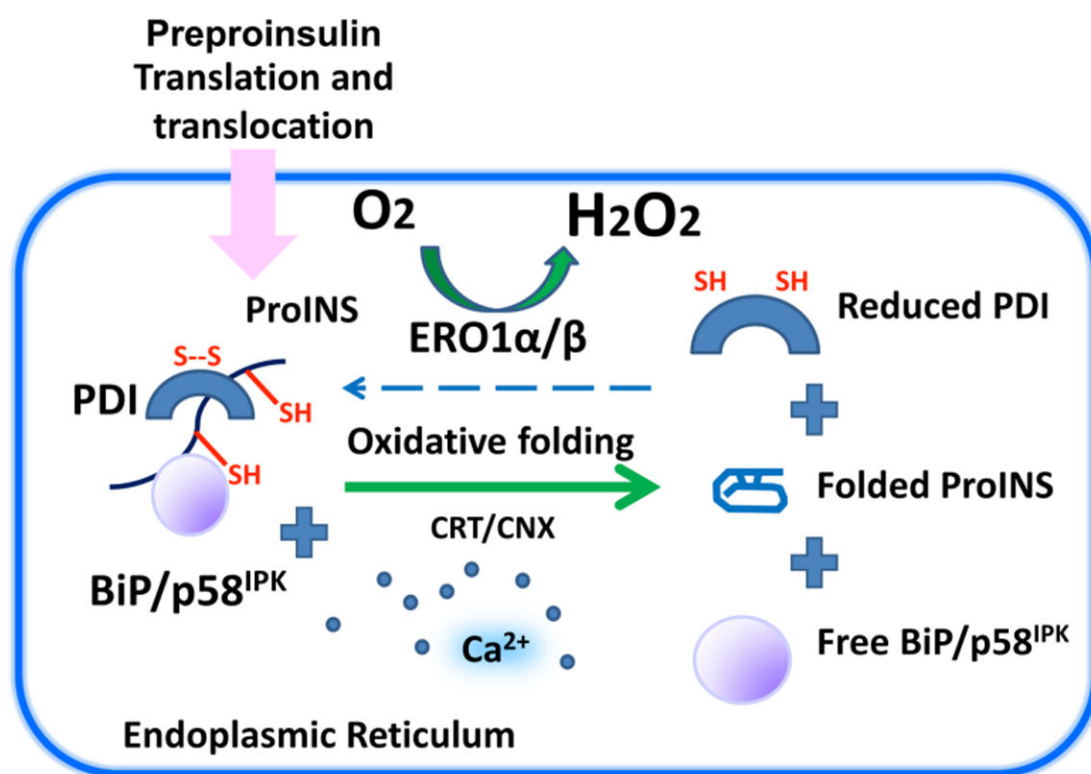


Figure 8. Proinsulin undergoes oxidative folding upon biosynthetic delivery into the ER lumen. Upon arrival in the ER lumen, proinsulin undergoes oxidative folding to form its three essential disulfide bonds, which is thought to be facilitated by members of the PDI family of ER oxidoreductases. The Ero1 family of upstream oxidases are considered important in shuttling the reducing equivalents (originating from proinsulin substrate) to molecular oxygen, while regenerating oxidized PDI proteins. In addition, Ca^{2+} dependent and – independent chaperones such as BiP and p58^{IPK} also play indispensable roles in assisting proinsulin folding, along with key ER resident proteins for other secretory protein substrates, such as calreticulin and calnexin.

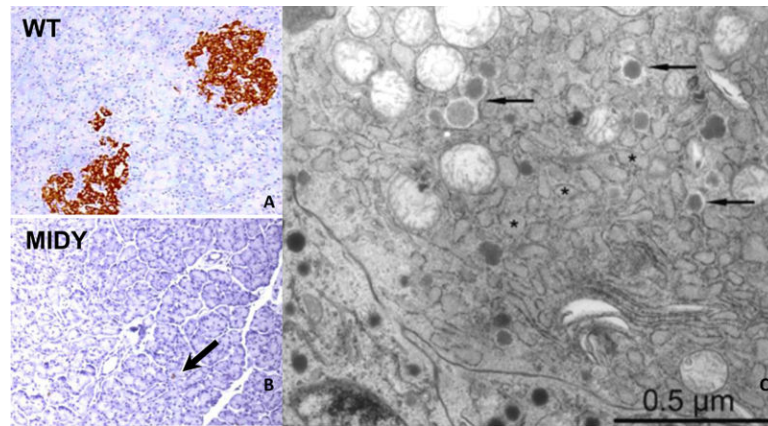


Figure 9: Pancreata and beta cells of transgenic MIDY pigs, reproduced from [226, 227]. (A) WT pig pancreas stained by immunoperoxidase with anti-insulin, counterstained with DAPI. (B) Two-year old transgenic MIDY pig pancreas stained as in panel A. (C) Transmission electron microscopy of pancreas from a 4.5-month old transgenic MIDY pig bearing only few beta cell secretory granules (arrows) and hypertrophied ER (asterisks).

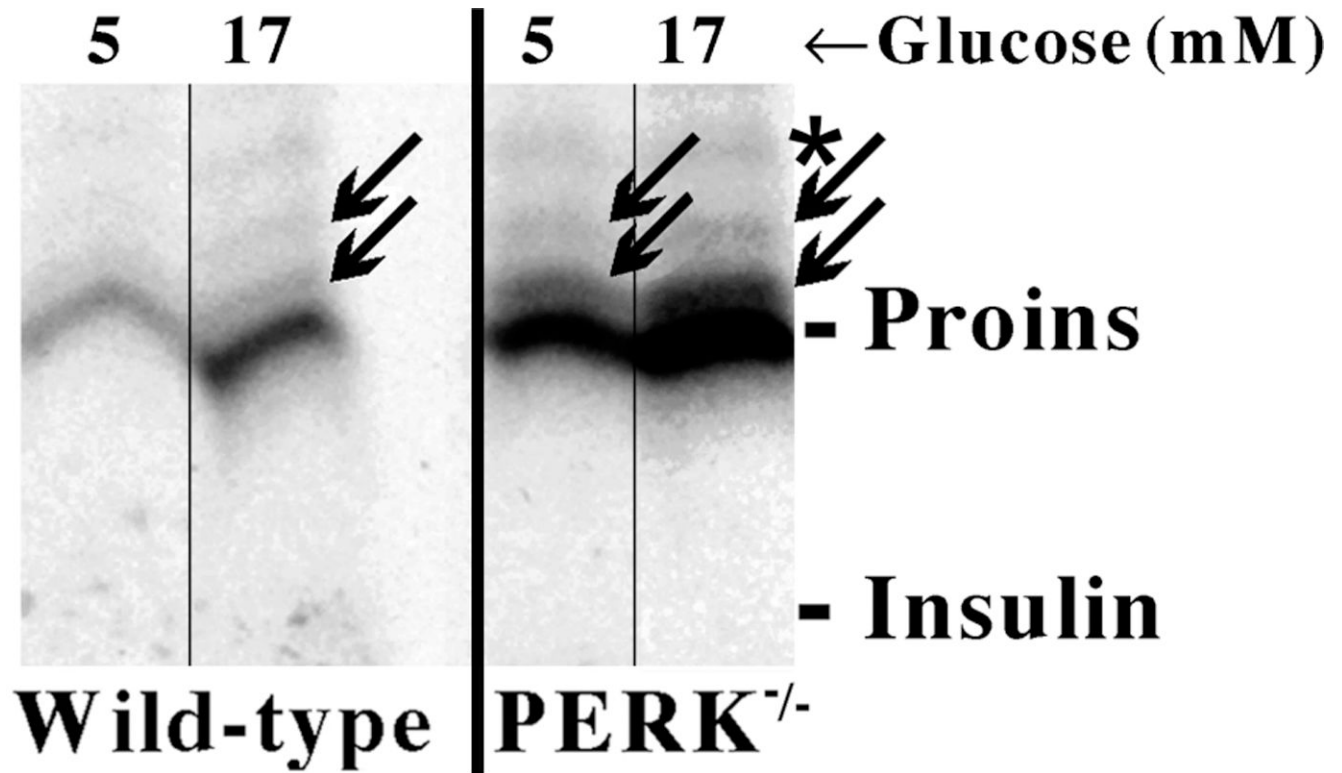


Figure 10: Two distinct nonnative intramolecular disulfide isomers of proinsulin (at arrows; the native isomer position is also indicated).

Isolated mouse pancreatic islets were preincubated for 40 min at the respective glucose concentrations, and were then pulse-labeled with ³⁵S-amino acids at these glucose concentrations. The islets were lysed, immunoprecipitated with anti-insulin, and analyzed by nonreducing Tris-tricine-urea-SDS-PAGE as in [93]. An asterisk identifies a proinsulin conversion intermediate, the processing of which takes place beyond the ER.

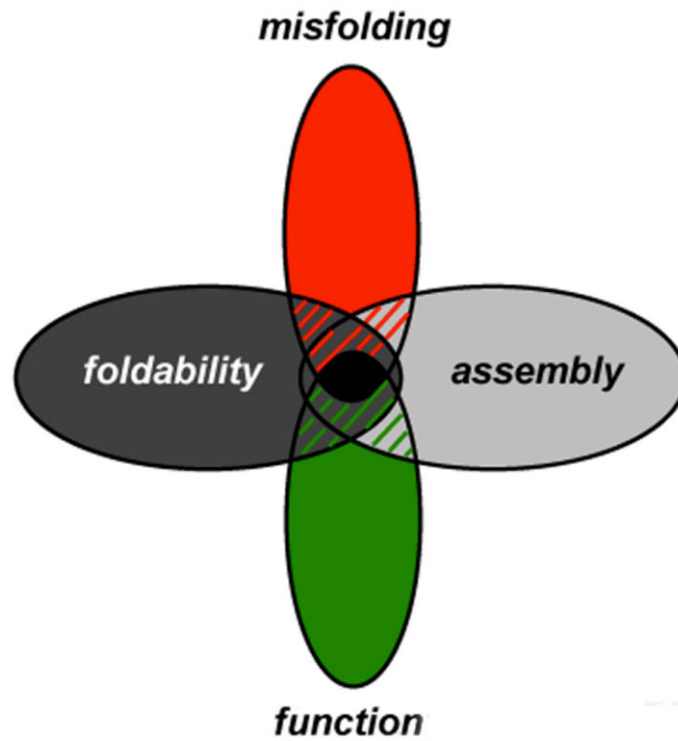


Figure 11. Evolution of insulin is constrained by multiple factors.

Venn diagram illustrating influence of protein misfolding, foldability and assembly as well as the traditional importance of direct biological function at the receptor level.

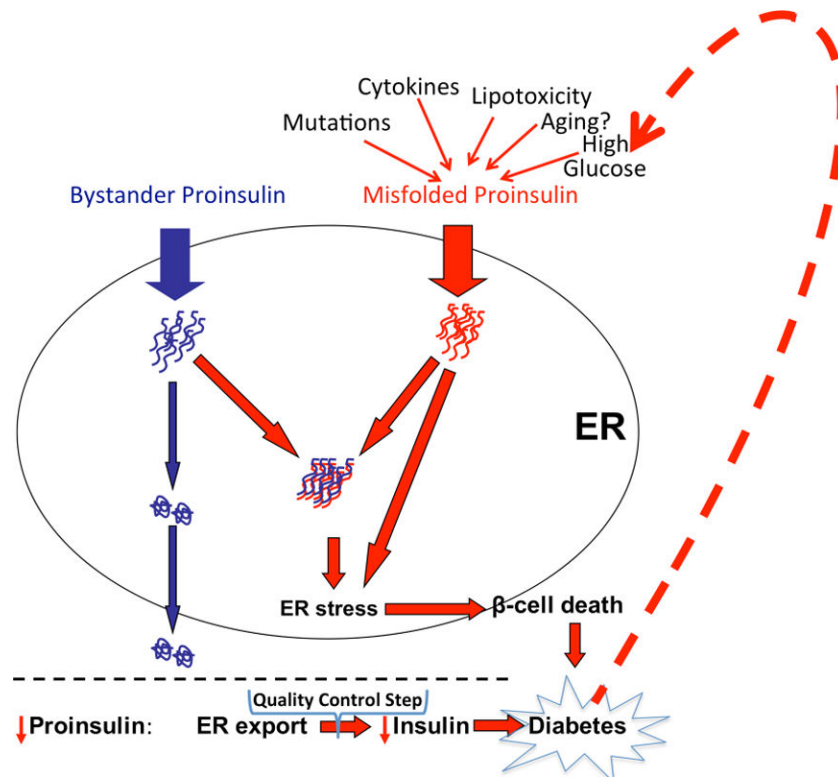


Figure 12: An hypothesis linking proinsulin misfolding to the pathogenesis of type 2 diabetes. Proinsulin must fold to become exported from the endoplasmic reticulum (ER) and make insulin (pathway in blue). Many factors may cause proinsulin misfolding (see text). Misfolded proinsulin molecules (in red) recruit bystander proinsulin molecules into complexes defective for ER export. Decreased export of proinsulin decreases insulin production and secretion leading to higher blood glucose. Hyperglycemia is a factor promoting additional biosynthesis of misfolded proinsulin creating a dangerous positive feedback loop that promotes diabetes. Ultimately, high level proinsulin misfolding can trigger ER stress and beta cell death.